PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/62, C07K 13/00 C12P 21/02

A1

(11) International Publication Number: WO 90/13653

(43) International Publication Date: 15 November 1990 (15.11.90)

(21) International Application Number: PCT/GB90/00650

(22) International Filing Date: 26 April 1990 (26.04.90)

(30) Priority data:
8909916.2 29 April 1989 (29.04.89) GB

(71) Applicant *(for all designated States except US)*: DELTA BIOTECHNOLOGY LIMITED [GB/GB]; Castle Court, Castle Boulevard, Nottingham NG7 1FD (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : BALLANCE, David, James [GB/GB]; 11 South Road, West Bridgford, Nottingham NG2 7AG (GB).

(74) Agent: BASSETT, Richard; Eric Potter & Clarkson, St Mary's Court, St Mary's Gate, Nottingham NG1 1LE (GB).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB, GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US.

+

Published
With international search report.

(54) Title: FUSION PROTEINS CONTAINING N-TERMINAL FRAGMENTS OF HUMAN SERUM ALBUMIN

(57) Abstract

A fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof, then the said polypeptide is one of various specified entities, including the 585 to 1578 portion of human fibronectin or a variant thereof. The HSA-like portion may have additional N-terminal residues, such as secretion leader sequences (signal sequences). The C-terminal portion is preferably the 585-1578 portion of human plasma fibronectin. The N-terminal and C-terminal portions may be cleavable to yield the isolated C-terminal portion, with the N-terminal portion having served to facilitate secretion from the host.

+ See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**ES**	Spain	**MC**	Monaco
AU	Australia	**FI**	Finland	**MG**	Madagascar
BB	Barbados	**FR**	France	**ML**	Mali
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Fasso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GR**	Greece	**NL**	Netherlands
BJ	Benin	**HU**	Hungary	**NO**	Norway
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**SD**	Sudan
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CG	Congo			**SN**	Senegal
CH	Switzerland	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
DE	Germany, Federal Republic of	**LK**	Sri Lanka	**TG**	Togo
DK	Denmark	**LU**	Luxembourg	**US**	United States of America

Fusion proteins containing N-terminal fragments of human serum albumin

The present invention relates to fusion polypeptides where two individual polypeptides or parts thereof are fused to form a single amino acid chain. Such fusion may arise from the expression of a single continuous coding sequence formed by recombinant DNA techniques.

Fusion polypeptides are known, for example those where a polypeptide which is the ultimately desired product of the process is expressed with an N-terminal "leader sequence" which encourages or allows secretion of the polypeptide from the cell. An example is disclosed in EP-A-116 201 (Chiron).

Human serum albumin (HSA) is a known protein found in the blood. EP-A-147 198 (Delta Biotechnology) discloses its expression in a transformed host, in this case yeast. Our earlier application EP-A-322 094 discloses N-terminal fragments of HSA, namely those consisting of residues 1-n where n is 369 to 419, which have therapeutic utility. The application also mentions the possibility of fusing the C-terminal residue of such molecules to other, unnamed, polypeptides.

One aspect of the present invention provides a fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor, or a variant thereof, (d) transforming growth factor, or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

The N-terminal portion of HSA is preferably the said 1-n portion, the 1-177 portion (up to and including the cysteine), the 1-200 portion (up to but excluding the cysteine) or a portion intermediate 1-177 and 1-200.

The term "human serum albumin" (HSA) is intended to include (but not necessarily to be restricted to) known or yet-to-be-discovered polymorphic forms of HSA. For example, albumin Naskapi has Lys-372 in place of Glu-372 and pro-albumin Christchurch has an altered pro-sequence. The term "variants" is intended to include (but not necessarily to be restricted to) minor artificial variations in sequence (such as molecules lacking one or a few residues, having conservative substitutions or minor insertions of residues, or having minor variations of amino acid structure). Thus polypeptides which have 80%, preferably 85%, 90%, 95% or 99%, homology with HSA are deemed to be "variants". It is also preferred for such variants to be physiologically equivalent to HSA; that is to say, variants preferably share at least one pharmacological utility with HSA. Furthermore, any putative variant which is to be used pharmacologically should be non-immunogenic in the animal (especially human) being treated.

Conservative substitutions are those where one or more amino acids are substituted for others having similar properties such that one skilled in the art of polypeptide chemistry would expect at least the secondary structure, and preferably the tertiary structure, of the polypeptide to be substantially unchanged. For example, typical such

substitutions include asparagine for glutamine, serine for asparagine and arginine for lysine. Variants may alternatively, or as well, lack up to ten (preferably only one or two) intermediate amino acid residues (ie not at the termini of the said N-terminal portion of HSA) in comparison with the corresponding portion of natural HSA; preferably any such omissions occur in the 100 to 369 portion of the molecule (relative to mature HSA itself) (if present). Similarly, up to ten, but preferably only one or two, amino acids may be added, again in the 100 to 369 portion for preference (if present). The term "physiologically functional equivalents" also encompasses larger molecules comprising the said sequence plus a further sequence at the N-terminal (for example, pro-HSA, pre-pro-HSA and met-HSA).

Clearly, the said "another polypeptide" in the fusion compounds of the invention cannot be the remaining portion of HSA, since otherwise the whole polypeptide would be HSA, which would not then be a "fusion polypeptide".

Even when the HSA-like portion is not the said 1-n portion of HSA, it is preferred for the non-HSA portion to be one of the said (a) to (h) entities.

The 1 to 368 portion of CD4 represents the first four disulphide-linked immunoglobulin-like domains of the human T lymphocyte CD4 protein, the gene for and amino acid sequence of which are disclosed in D. Smith et al (1987) Science 328, 1704-1707. It is used to combat HIV infections.

The sequence of human platelet-derived growth factor (PDGF) is described in Collins et al (1985) Nature 316, 748-750. Similarly, the sequence of transforming growth factors β (TGF-β) is described in Derynck et al (1985) Nature 316, 701-705. These growth factors are useful for wound-healing.

A cDNA sequence for the 1-261 portion of Fn was disclosed in EP-A-207 751 (obtained from plasmid pFH6 with endonuclease PvuII). This portion binds fibrin and can be used to direct fused compounds to blood clots.

A cDNA sequence for the 278-578 portion of Fn, which contains a collagen-binding domain, was disclosed by R.J. Owens and F.E. Baralle in 1986 E.M.B.O.J. 5, 2825-2830. This portion will bind to platelets.

The 1-272 portion of von Willebrand's Factor binds and stabilises factor VIII. The sequence is given in Bontham et al, Nucl. Acids Res. 14, 7125-7127.

Variants of alpha-1-antitrypsin include those disclosed by Rosenburg et al (1984) Nature 312, 77-80. In particular, the present invention includes the Pittsburgh variant (Met^{358} is mutated to Arg) and the variant where Pro^{357} and Met^{358} are mutated to alanine and arginine respectively. These compounds are useful in the treatment of septic shock and lung disorders.

Variants of the non-HSA portion of the polypeptides of the invention include variations as discussed above in relation to the HSA portion, including those with conservative amino acid substitutions, and also homologues from other species.

The fusion polypeptides of the invention may have N-terminal amino acids which extend beyond the portion corresponding to the N-terminal portion of HSA. For example, if the HSA-like portion corresponds to an N-terminal portion of mature HSA, then pre-, pro-, or pre-pro sequences may be added thereto, for example the yeast alpha-factor leader sequence. The fused leader portions of WO 90/01063 may be used. The polypeptide which is

fused to the HSA portion may be a naturally-occurring polypeptide, a fragment thereof or a novel polypeptide, including a fusion polypeptide. For example, in Example 3 below, a fragment of fibronectin is fused to the HSA portion via a 4 amino acid linker.

It has been found that the amino terminal portion of the HSA molecule is so structured as to favour particularly efficient translocation and export of the fusion compounds of the invention in eukaryotic cells.

A second aspect of the invention provides a transformed host having a nucleotide sequence so arranged as to express a fusion polypeptide as described above. By "so arranged", we mean, for example, that the nucleotide sequence is in correct reading frame with an appropriate RNA polymerase binding site and translation start sequence and is under the control of a suitable promoter. The promoter may be homologous with or heterologous to the host. Downstream (3') regulatory sequences may be included if desired, as is known. The host is preferably yeast (for example <u>Saccharomyces</u> spp., e.g. <u>S. cerevisiae</u>; <u>Kluyveromyces</u> spp., e.g. <u>K. lactis</u>; Pichia spp.; or <u>Schizosaccharomyces</u> spp., e.g. <u>S. pombe</u>) but may be any

other suitable host such as *E. coli*, *B. subtilis*, *Aspergillus* spp., mammalian cells, plant cells or insect cells.

A third aspect of the invention provides a process for preparing a fusion polypeptide according to the first aspect of the invention by cultivation of a transformed host according to the second aspect of the invention, followed by separation of the fusion polypeptide in a useful form.

A fourth aspect of the invention provides therapeutic methods of treatment of the human or other animal body comprising administration of such a fusion polypeptide.

In the methods of the invention we are particularly concerned to improve the efficiency of secretion of useful therapeutic human proteins from yeast and have conceived the idea of fusing to amino-terminal portions of HSA those proteins which may ordinarily be only inefficiently secreted. One such protein is a potentially valuable wound-healing polypeptide representing amino acids 585 to 1578 of human fibronectin (referred to herein as Fn 585-1578). As we have described in a separate application (filed simultaneously herewith) this molecule contains cell spreading, chemotactic and chemokinetic activities

useful in healing wounds. The fusion polypeptides of the present invention wherein the C-terminal portion is Fn 585-1578 can be used for wound healing applications as biosynthesised, especially where the hybrid human protein will be topically applied. However, the portion representing amino acids 585 to 1578 of human fibronectin can if desired be recovered from the fusion protein by preceding the first amino acid of the fibronectin portion by amino acids comprising a factor X cleavage site. After isolation of the fusion protein from culture supernatant, the desired molecule is released by factor X cleavage and purified by suitable chromatography (e.g. ion-exchange chromatography). Other sites providing for enzymatic or chemical cleavage can be provided, either by appropriate juxtaposition of the N-terminal and C-terminal portions or by the insertion therebetween of an appropriate linker.

At least some of the fusion polypeptides of the invention, especially those including the said CD4 and vWF fragments, PDGF and α_1AT, also have an increased half-life in the blood and therefore have advantages and therapeutic utilities themselves, namely the therapeutic utility of the non-HSA portion of the molecule. In the case of α_1AT and others, the compound will normally be administered as

a one-off dose or only a few doses over a short period, rather than over a long period, and therefore the compounds are less likely to cause an immune response.

EXAMPLES : SUMMARY

Standard recombinant DNA procedures were as described by Maniatis et al (1982 and recent 2nd edition) unless otherwise stated. Construction and analysis of phage M13 recombinant clones was as described by Messing (1983) and Sanger et al (1977).

DNA sequences encoding portions of human serum albumin used in the construction of the following molecules are derived from the plasmids mHOB12 and pDBD2 (EP-A-322 094, Delta Biotechnology Ltd, relevant portions of which are reproduced below) or by synthesis of oligonucleotides equivalent to parts of this sequence. DNA sequences encoding portions of human fibronectin are derived from the plasmid pFHDEL1, or by synthesis of oligonucleotides equivalent to parts of this sequence. Plasmid pFHDEL1, which contains the complete human cDNA encoding plasma fibronectin, was obtained by ligation of DNA derived from plasmids pFH6, 16, 54, 154 and 1 (EP-A-207 751; Delta Biotechnology Ltd).

This DNA represents an mRNA variant which does not contain the 'ED' sequence and had an 89-amino acid variant of the III-CS region (R.J. Owens, A.R. Kornblihtt and F.E. Baralle (1986) Oxford Surveys on Eukaryotic Genes 3 141-160). The map of this vector is disclosed in Fig. 11 and the protein sequence of the mature polypeptide produced by expression of this cDNA is shown in Fig. 5.

Oligonucleotides were synthesised on an Applied Biosystems 380B oligonucleotide synthesiser according to the manufacturer's recommendations (Applied Biosystems, Warrington, Cheshire, UK).

An expression vector was constructed in which DNA encoding the HSA secretion signal and mature HSA up to and including the 387th amino acid, leucine, fused in frame to DNA encoding a segment of human fibronectin representing amino acids 585 to 1578 inclusive, was placed downstream of the hybrid promoter of EP-A-258 067 (Delta Biotechnology), which is a highly efficient galactose-inducible promoter functional in Saccharomyces cerevisiae. The codon for the 1578th amino acid of human fibronectin was directly followed by a stop codon (TAA) and then the S. cerevisiae phosphoglycerate kinase (PGK) gene transcription terminator. This vector was then introduced into S. cerevisiae by transformation, wherein it directed

the expression and secretion from the cells of a hybrid molecule representing the N-terminal 387 amino acids of HSA C-terminally fused to amino acids 585 to 1578 of human fibronectin.

In a second example a similar vector is constructed so as to enable secretion by S. cerevisiae of a hybrid molecule representing the N-terminal 195 amino acids of HSA C-terminally fused to amino acids 585 to 1578 of human fibronectin.

Aspects of the present invention will now be described by way of example and with reference to the accompanying drawings, in which:

Figure 1 (on two sheets) depicts the amino acid sequence currently thought to be the most representative of natural HSA, with (boxed) the alternative C-termini of HSA(1-n);

Figure 2 (on two sheets) depicts the DNA sequence coding for mature HSA, wherein the sequence included in Linker 3 is underlined;

Figure 3 illustrates, diagrammatically, the construction of mHOB16;

Figure 4 illustrates, diagrammatically, the construction of pHOB31;

Figure 5 (on 6 sheets) illustrates the mature protein sequence encoded by the Fn plasmid pFHDEL1;

Figure 6 illustrates Linker 5, showing the eight constituent oligonucleotides;

Figure 7 shows schematically the construction of plasmid pDBDF2;

Figure 8 shows schematically the construction of plasmid pDBDF5;

Figure 9 shows schematically the construction of plasmid pDBDF9;

Figure 10 shows schematically the construction of plasmid DBDF12, using plasmid pFHDEL1; and

Figure 11 shows a map of plasmid pFHDEL1.

EXAMPLE 1 : HSA 1-387 FUSED TO Fn 585-1578

The following is an account of a preparation of plasmids comprising sequences encoding a portion of HSA, as is disclosed in EP-A-322 094.

The human serum albumin coding sequence used in the construction of the following molecules is derived from the plasmid M13mp19.7 (EP-A-201 239, Delta Biotech- nology Ltd.) or by synthesis of oligonucleotides equivalent to parts of this sequence. Oligonucleotides were synthesised using phosphoramidite chemistry on an Applied Biosystems 380B oligonucleotide synthesizer according to the manufacturer's recommendations (AB Inc., Warrington, Cheshire, England).

An oligonucleotide was synthesised (Linker A) which represented a part of the known HSA coding sequence (Figure 2) from the PstI site (1235-1240, Figure 2) to the codon for valine 381 wherein that codon was changed from GTG to GTC:

Linker 1

	D	P	H	E	C	Y	
5′	GAT	CCT	CAT	GAA	TGC	TAT	
3′ ACGT	CTA	GGA	GTA	CTT	ACG	ATA	
			1247				
A	K	V	F	D	E	F	K
GCC	AAA	GTG	TTC	GAT	GAA	TTT	AAA
CGG	TTT	CAC	AAG	CTA	CTT	AAA	TTT
		1267					
P	L	V					
CTT	GTC	3′					
GGA	CAG	5′					

Linker 1 was ligated into the vector M13mp19 (Norrander et al, 1983) which had been digested with PstI and HincII and the ligation mixture was used to transfect E.coli strain XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Recombinant clones were identified by their failure to evolve a blue colour on medium containing the chromogenic indicator X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in the present of IPTG (isopropylthio-β-galactoside). DNA sequence analysis of template DNA prepared from bacteriophage particles of recombinant clones identified a molecule with the required DNA sequence, designated mHOB12 (Figure 3).

M13mp19.7 consists of the coding region of mature HSA in M13mp19 (Norrander et al, 1983) such that the codon for the first amino acid of HSA, GAT, overlaps a unique XhoI site thus:

```
                    Asp   Ala
5'      C T C G A G A T G C A      3'
3'      G A G C T C T A C G T      5'
           XhoI
```

(EP-A-210 239). M13mp19.7 was digested with XhoI and made flush-ended by S1-nuclease treatment and was then ligated with the following oligonucleotide (Linker 2):

Linker 2

```
5' T C T T T T A T C C A A G C T T G G A T A A A A G A  3'
3' A G A A A A T A G G T T C G A A C C T A T T T T C T  5'
                   HindIII
```

The ligation mix was then used to transfect E.coli XL1-Blue and template DNA was prepared from several plaques and then analysed by DNA sequencing to identify a clone, pDBD1 (Figure 4), with the correct sequence.

A 1.1 kb HindIII to PstI fragment representing the 5′ end of the HSA coding region and one half of the inserted oligonucleotide linker was isolated from pDBD1 by agarose gel electrophoresis. This fragment was then ligated with double stranded mHOB12 previously digested with HindIII and PstI and the ligation mix was then used to transfect *E.coli* XL1-Blue. Single stranded template DNA was prepared from mature bacteriophage particles of several plaques. The DNA was made double stranded *in vitro* by extension from annealed sequencing primer with the Klenow fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates. Restriction enzyme analysis of this DNA permitted the identification of a clone with the correct configuration, mHOB15 (Figure 4).

The following oligonucleotide (Linker 3) represents from the codon for the 382nd amino acid of mature HSA (glutamate, GAA) to the codon for lysine 389 which is followed by a stop codon (TAA) and a HindIII site and then a BamHI cohesive end:

Linker 3

```
    E   E   P   Q   N   L   I   K   J
5′ GAA GAG CCT CAG AAT TTA ATC AAA TAA GCTTG       3′
3′ CTT CTC GGA GTC TTA AAT TAG TTT ATT CGAACCTAG  5′
```

This was ligated into double stranded mHOB15, previously digested with HincII and BamHI. After ligation, the DNA was digested with HincII to destroy all non-recombinant molecules and then used to transfect E.coli XL1-Blue. Single stranded DNA was prepared from bacteriophage particles of a number of clones and subjected to DNA sequence analysis. One clone having the correct DNA sequence was designated mHOB16 (Figure 4).

A molecule in which the mature HSA coding region was fused to the HSA secretion signal was created by insertion of Linker 4 into BamHI and XhoI digested M13mp19.7 to form pDBD2 (Figure 4).

Linker 4

	M	K	W	V	S	F
5′ GATCC	ATG	AAG	TGG	GTA	AGC	TTT
G	TAC	TTC	ACC	CAT	TCG	AAA

I	S	L	L	F	L	F	S
ATT	TCC	CTT	CTT	TTT	CTC	TTT	AGC
TAA	AGG	GAA	GAA	AAA	GAG	AAA	TCG

S	A	Y	S	R	G	V	F
TCG	GCT	TAT	TCC	AGG	GGT	GTG	TTT
AGC	CGA	ATA	AGG	TCC	CCA	CAC	AAA

R	R
CG	3′
GCAGCT	5′

In this linker the codon for the fourth amino acid after the initial methionine, ACC for threonine in the HSA pre-pro leader sequence (Lawn et al, 1981), has been changed to AGC for serine to create a HindIII site.

A sequence of synthetic DNA representing a part of the known HSA coding sequence (Lawn et al., 1981) (amino acids 382 to 387, Fig. 2), fused to part of the known fibronectin coding sequence (Kornblihtt et al., 1985) (amino acids 585 to 640, Fig. 2), was prepared by synthesising six oligonucleotides (Linker 5, Fig. 6). The oligonucleotides 2, 3, 4, 6, 7 and 8 were phosphorylated using T4 polynucleotide kinase and then the oligonucleotides were annealed under standard conditions in pairs, i.e. 1+8, 2+7, 3+6 and 4+5. The annealed oligonucleotides were then mixed together and ligated with mHOB12 which had previously been digested with the restriction enzymes HincII and EcoRI. The ligation

mixture was then used to transfect E.coli XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Single stranded template DNA was then prepared from mature bacteriophage particles derived from several independent plaques and then was analysed by DNA sequencing. A clone in which a linker of the expected sequence had been correctly inserted into the vector was designated pDBDF1 (Fig. 7). This plasmid was then digested with PstI and EcoRI and the approx. 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 (Fig. 7) and BamHI + EcoRI digested pUC19 (Yanisch-Perron, et al., 1985) to form pDBDF2 (Fig. 7).

A plasmid containing a DNA sequence encoding full length human fibronectin, pFHDEL1, was digested with EcoRI and XhoI and a 0.77kb EcoRI-XhoI fragment (Fig. 8) was isolated and then ligated with EcoRI and SalI digested M13 mp18 (Norrander et al., 1983) to form pDBDF3 (Fig. 8).

The following oligonucleotide linker (Linker 6) was synthesised, representing from the PstI site at 4784-4791 of the fibronectin sequence of EP-A-207 751 to the codon for tyrosine 1578 (Fig. 5) which is followed by a stop codon (TAA), a HindIII site and then a BamHI cohesive end:

Linker 6

```
        G   P   D   Q   T   E   M   T   I   E   G   L
       GGT CCA GAT CAA ACA GAA ATG ACT ATT GAA GGC TTG
     A CGT CCA GGT CTA GTT TGT CTT TAC TGA TAA CTT CCG AAC

  Q   P   T   V   E   Y     Stop
 CAG CCC ACA GTG GAG TAT    TAA   GCTTG
 GTC GGG TGT CAC CTC ATA    ATT   CGAACCTAG
```

This linker was then ligated with PstI and HindIII digested pDBDF3 to form pDBDF4 (Fig. 8). The following DNA fragments were then ligated together with BglII digested pKV50 (EP-A-258 067) as shown in Fig. 8: 0.68kb EcoRI-BamHI fragment of pDBDF4, 1.5kb BamHI-StuI fragment of pDBDF2 and the 2.2kb StuI-EcoRI fragment of pFHDEL1. The resultant plasmid pDBDF5 (Fig. 8) includes the promoter of EP-A-258 067 to direct the expression of the HSA secretion signal fused to DNA encoding amino acids 1-387 of mature HSA, in turn fused directly and in frame with DNA encoding amino acids 585-1578 of human fibronectin, after which translation would terminate at the stop codon TAA. This is then followed by the *S.cerevisiae* PGK gene transcription terminator. The

plasmid also contains sequences which permit selection and maintenance in Escherichia coli and S.cerevisiae (EP-A-258 067).

This plasmid was introduced into S.cerevisiae S150-2B (leu2-3 leu2-112 ura3-52 trp1-289 his3- 1) by standard procedures (Beggs, 1978). Transformants were subsequently analysed and found to produce the HSA-fibronectin fusion protein.

EXAMPLE 2 : HSA 1-195 FUSED TO Fn 585-1578

In this second example the first domain of human serum albumin (amino acids 1-195) is fused to amino acids 585-1578 of human fibronectin.

The plasmid pDBD2 was digested with BamHI and BglII and the 0.79kb fragment was purified and then ligated with BamHI-digested M13mp19 to form pDBDF6 (Fig. 6). The following oligonucleotide:

5'-C C A A A G C T C G A G G A A C T T C G-3'

was used as a mutagenic primer to create a XhoI site in pDBDF6 by in vitro mutagenesis using a kit supplied by Amersham International PLC. This site was created by

changing base number 696 of HSA from a T to a G (Fig. 2). The plasmid thus formed was designated pDBDF7 (Fig. 9). The following linker was then synthesised to represent from this newly created XhoI site to the codon for lysine 195 of HSA (AAA) and then from the codon for isoleucine 585 of fibronectin to the ends of oligonucleotides 1 and 8 shown in Fig. 6.

Linker 7

```
    D   E   L   R   D   E   G   K   A   S   S   A   K
TC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA
      A CTT GAA GCC CTA CTT CCC TTC CGA AGC AGA CGG TTT

    I   T   E   T   P   S   Q   P   N   S   H
   ATC ACT GAG ACT CCG AGT CAG C
   TAG TGA CTC TGA GGC TCA GTC GGG TTG AGG GTG G
```

This linker was ligated with the annealed oligonucleotides shown in Fig. 3, i.e. 2+7, 3+6 and 4+5 together with XhoI and EcoRI digested pDBDF7 to form pDBDF8 (Fig. 9). Note that in order to recreate the original HSA DNA sequence, and hence amino acid sequence, insertion of linker 7 and the other oligonucleotides into pDBDF7 does not recreate the XhoI site.

The 0.83kb BamHI-StuI fragment of pDBDF8 was purified and then was ligated with the 0.68kb EcoRI-BamHI fragment of pDBDF2 and the 2.22kb StuI-EcoRI fragment of pFHDEL1 into BglII-digested pKV50 to form pDBDF9 (Fig. 9). This plasmid is similar to pDBDF5 except that it specifies only residues 1-195 of HSA rather than 1-387 as in pDBDF5.

When introduced into *S.cerevisiae* S150-2B as above, the plasmid directed the expression and secretion of a hybrid molecule composed of residues 1-195 of HSA fused to residues 585-1578 of fibronectin.

EXAMPLE 3 : HSA 1-387 FUSED TO Fn 585-1578, AS CLEAVABLE MOLECULE

In order to facilitate production of large amounts of residues 585-1578 of fibronectin, a construct was made in which DNA encoding residues 1-387 of HSA was separated from DNA encoding residues 585-1578 of fibronectin by the sequence

```
 I   E   G   R
ATT GAA GGT AGA
TAA CTT CCA TCT
```

which specifies the cleavage recognition site for the blood clotting Factor X. Consequently the purified secreted product can be treated with Factor X and then the fibronectin part of the molecule can be separated from the HSA part.

To do this two oligonucleotides were synthesised and then annealed to form Linker 8.

Linker 8

E	E	P	Q	N	L	I	E	G
GAA	GAG	CCT	CAG	AAT	TTA	ATT	GAA	GGT
CTT	CTC	GGA	GTC	TTA	AAT	TAA	CTT	CCA
R	I	T	E	T	P	S	Q	P
AGA	ATC	ACT	GAG	ACT	CCG	AGT	CAG	C
TCT	TAG	TGA	CTC	TGA	GGC	TCA	GTC	GGG
N	S	H						
TTG	AGG	GTG G						

This linker was then ligated with the annealed oligonucleotides shown in Fig. 6, i.e. 2+7, 3+6 and 4+5 into HincII andEcoRI digested mHOB12, to form pDBDF10

(Fig. 7). The plasmid was then digested with PstI and EcoRI and the roughly 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 and BamHI and EcoRI digested pUC19 to form pDBDF11 (Fig. 10).

The 1.5kb BamHI-StuI fragment of pDBDF11 was then ligated with the 0.68kb EcoRI-BamH1 fragment of pDBDF4 and the 2.22kb StuI-EcoRI fragment of pFHDEL1 into BglII-digested pKV50 to form pDBDF12 (Fig. 10). This plasmid was then introduced into *S.cerevisiae* S150-2B. The purified secreted fusion protein was treated with Factor X to liberate the fibronectin fragment representing residues 585-1578 of the native molecule.

REFERENCES

Beggs, J.D. (1978) Nature 275, 104-109

Kornblihtt et al. (1985) EMBO J. 4, 1755-1759

Lawn, R.M. et al. (1981) Nucl. Acid. Res. 9, 6103-6114

Maniatis, T. et al. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Messing, J. (1983) Methods Enzymol. 101, 20-78

Norrander, J. et al. (1983) Gene 26, 101-106

Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467

Yanisch-Perron, C. (1985) Gene 33, 103-119

CLAIMS

1. A fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor or a variant thereof, (d) transforming growth factor β or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

2. A fusion polypeptide according to Claim 1 additionally comprising at least one N-terminal amino acid extending beyond the portion corresponding to the N-terminal portion of HSA.

3. A fusion polypeptide according to Claim 1 or 2 wherein there is a cleavable region at the junction of the said N-terminal or C-terminal portions.

4. A fusion polypeptide according to any one of the preceding claims wherein the said C-terminal portion is the 585 to 1578 portion of human plasma fibronectin or a variant thereof.

5. A transformed or transfected host having a nucleotide sequence so arranged as to express a fusion polypeptide according to any one of the preceding claims.

6. A process for preparing a fusion polypeptide by cultivation of a host according to Claim 5, followed by separation of the fusion polypeptide in a useful form.

7. A fusion polypeptide according to any one of Claims 1 to 4 for use in therapy.

FIGURE 1

10 20
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys

30 40
Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val

50 60
Lys Leu Val Asn Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu

70 80
Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu

90 100
Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro Glu Arg Asn Glu

110 120
Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val

130 140
Asp Val Met Cys Thr Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr

150 160
Glu Ile Ala Arg Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg

170 180
Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala Cys Leu Leu Pro

190 200
Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys

210 220
Ala Ser Leu Gln Lys Phe Gly Glu Arg. Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser

230 240
Gln Arg Phe Pro Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys

250 260
Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu

270 280
Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu

290 300
Lys Pro Leu Leu Glu Lys Ser His Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala

310 320
Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala

330 340
Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg Arg His Pro Asp

350 360
Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr Tyr Glu Thr Thr Leu Glu Lys Cys

370 380
Cys Ala Ala Ala Asp Pro His Glu Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu

FIGURE 1 Cont.

									390										400
Val	Glu	Glu	Pro	Gln	Asn	Leu	Ile	Lys	Gln	Asn	Cys	Glu	Leu	Phe	Glu	Gln	Leu	Gly	Glu
									410										420
Tyr	Lys	Phe	Gln	Asn	Ala	Leu	Leu	Val	Arg	Tyr	Thr	Lys	Lys	Val	Pro	Gln	Val	Ser	Thr
									430										440
Pro	Thr	Leu	Val	Glu	Val	Ser	Arg	Asn	Leu	Gly	Lys	Val	Gly	Ser	Lys	Cys	Cys	Lys	His
									450										460
Pro	Glu	Ala	Lys	Arg	Met	Pro	Cys	Ala	Glu	Asp	Tyr	Leu	Ser	Val	Val	Leu	Asn	Gln	Leu
									470										480
Cys	Val	Leu	His	Glu	Lys	Thr	Pro	Val	Ser	Asp	Arg	Val	Thr	Lys	Cys	Cys	Thr	Glu	Ser
									490										500
Leu	Val	Asn	Arg	Arg	Pro	Cys	Phe	Ser	Ala	Leu	Glu	Val	Asp	Glu	Thr	Tyr	Val	Pro	Lys
									510										520
Glu	Phe	Asn	Ala	Glu	Thr	Phe	Thr	Phe	His	Ala	Asp	Ile	Cys	Thr	Leu	Ser	Glu	Lys	Glu
									530										540
Arg	Gln	Ile	Lys	Lys	Gln	Thr	Ala	Leu	Val	Glu	Leu	Val	Lys	His	Lys	Pro	Lys	Ala	Thr
									550										560
Lys	Glu	Gln	Leu	Lys	Ala	Val	Met	Asp	Asp	Phe	Ala	Ala	Phe	Val	Glu	Lys	Cys	Cys	Lys
									570										580
Ala	Asp	Asp	Lys	Glu	Thr	Cys	Phe	Ala	Glu	Glu	Gly	Lys	Lys	Leu	Val	Ala	Ala	Ser	Gln
Ala	Ala	Leu	Gly	Leu															

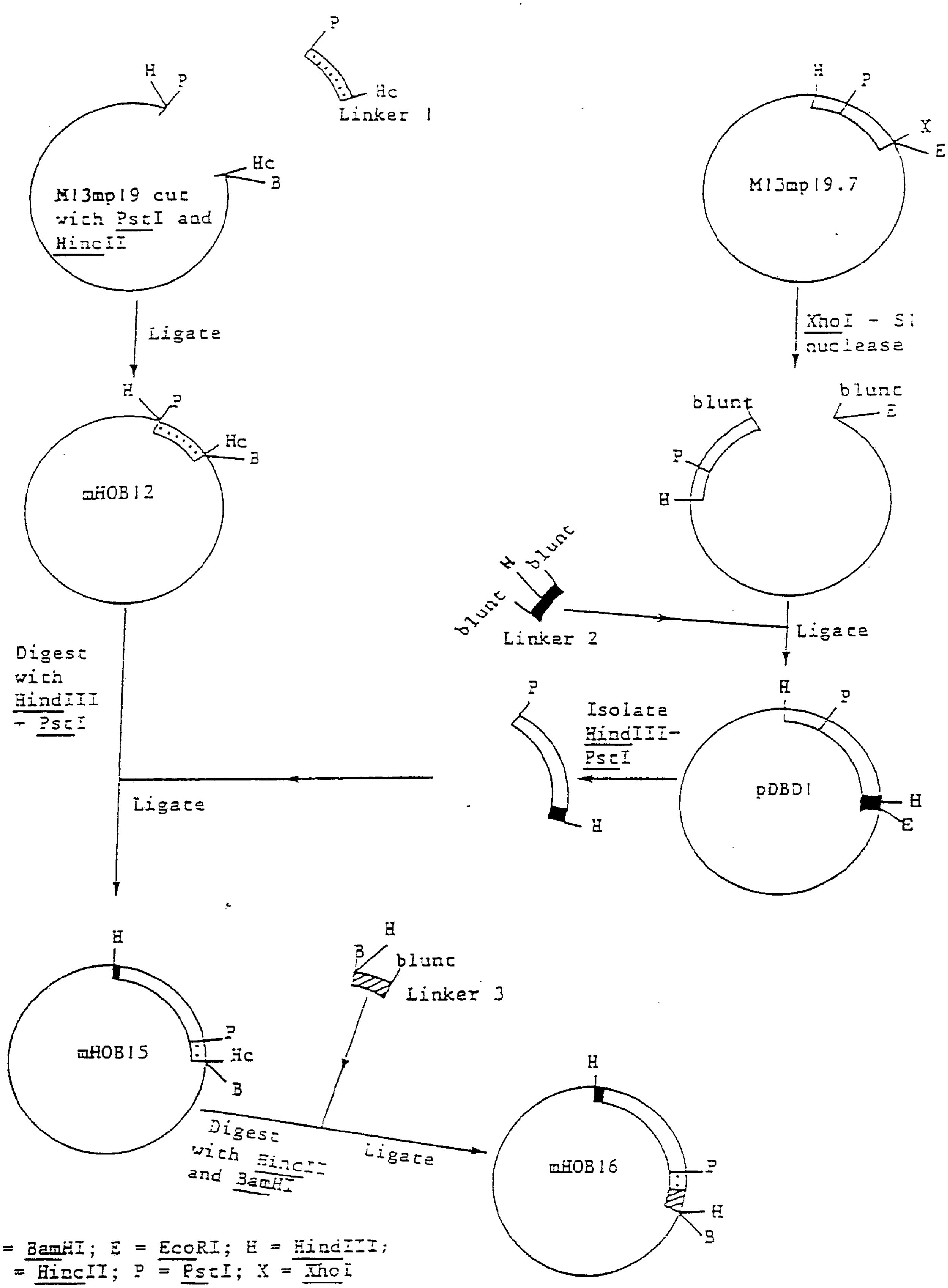
FIGURE 2 DNA sequence coding for mature HSA

```
        10        20        30        40        50        60        70        80
GATGCACACAAGAGTGAGGTTGCTCATCGGTTTAAAGATTTGGGAGAAGAAAATTTCAAAGCCTTGGTGTTGATTGCCTT
 D  A  H  K  S  E  V  A  H  R  F  K  D  L  G  E  E  N  F  K  A  L  V  L  I  A  F

        90       100       110       120       130       140       150       160
TGCTCAGTATCTTCAGCAGTGTCCATTTGAAGATCATGTAAAATTAGTGAATGAAGTAACTGAATTTGCAAAAACATGTG
  A  Q  Y  L  Q  Q  C  P  F  E  D  H  V  K  L  V  N  E  V  T  E  F  A  K  T  C

       170       180       190       200       210       220       230       240
TTGCTGATGAGTCAGCTGAAAATTGTGACAAATCACTTCATACCCTTTTTGGAGACAAATTATGCACAGTTGCAACTCTT
V  A  D  E  S  A  E  N  C  D  K  S  L  H  T  L  F  G  D  K  L  C  T  V  A  T  L

       250       260       270       280       290       300       310       320
CGTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAACCTGAGAGAAATGAATGCTTCTTGCAACACAAAGA
 R  E  T  Y  G  E  M  A  D  C  C  A  K  Q  E  P  E  R  N  E  C  F  L  Q  H  K  D

       330       340       350       360       370       380       390       400
TGACAACCCAAACCTCCCCCGATTGGTGAGACCAGAGGTTGATGTGATGTGCACTGCTTTTCATGACAATGAAGAGACAT
  D  N  P  N  L  P  R  L  V  R  P  E  V  D  V  M  C  T  A  F  H  D  N  E  E  T

       410       420       430       440       450       460       470       480
TTTTGAAAAAATACTTATATGAAATTGCCAGAAGACATCCTTACTTTTATGCCCCGGAACTCCTTTTCTTTGCTAAAAGG
F  L  K  K  Y  L  Y  E  I  A  R  R  H  P  Y  F  Y  A  P  E  L  L  F  F  A  K  R

       490       500       510       520       530       540       550       560
TATAAAGCTGCTTTTACAGAATGTTGCCAAGCTGCTGATAAAGCTGCCTGCCTGTTGCCAAAGCTCGATGAACTTCGGGA
 Y  K  A  A  F  T  E  C  C  Q  A  A  D  K  A  A  C  L  L  P  K  L  D  E  L  R  D

       570       580       590       600       610       620       630       640
TGAAGGGAAGGCTTCGTCTGCCAAACAGAGACTCAAATGTGCCAGTCTCCAAAAATTTGGAGAAAGAGCTTTCAAAGCAT
  E  G  K  A  S  S  A  K  Q  R  L  K  C  A  S  L  Q  K  F  G  E  R  A  F  K  A

       650       660       670       680       690       700       710       720
GGGCAGTGGCTCGCCTGAGCCAGAGATTTCCCAAAGCTGAGTTTGCAGAAGTTTCCAAGTTAGTGACAGATCTTACCAAA
W  A  V  A  R  L  S  Q  R  F  P  K  A  E  F  A  E  V  S  K  L  V  T  D  L  T  K

       730       740       750       760       770       780       790       800
GTCCACACGGAATGCTGCCATGGAGATCTGCTTGAATGTGCTGATGACAGGGCGGACCTTGCCAAGTATATCTGTGAAAA
 V  H  T  E  C  C  H  G  D  L  L  E  C  A  D  D  R  A  D  L  A  K  Y  I  C  E  N

       810       820       830       840       850       860       870       880
TCAGGATTCGATCTCCAGTAAACTGAAGGAATGCTGTGAAAAACCTCTGTTGGAAAAATCCCACTGCATTGCCGAAGTGG
  Q  D  S  I  S  S  K  L  K  E  C  C  E  K  P  L  L  E  K  S  H  C  I  A  E  V

       890       900       910       920       930       940       950       960
AAAATGATGAGATGCCTGCTGACTTGCCTTCATTAGCTGCTGATTTTGTTGAAAGTAAGGATGTTTGCAAAAACTATGCT
E  N  D  E  M  P  A  D  L  P  S  L  A  A  D  F  V  E  S  K  D  V  C  K  N  Y  A

       970       980       990      1000      1010      1020      1030      1040
GAGGCAAAGGATGTCTTCCTGGGCATGTTTTTGTATGAATATGCAAGAAGGCATCCTGATTACTCTGTCGTGCTGCTGCT
 E  A  K  D  V  F  L  G  M  F  L  Y  E  Y  A  R  R  H  P  D  Y  S  V  V  L  L  L
```

FIGURE 2 Cont.

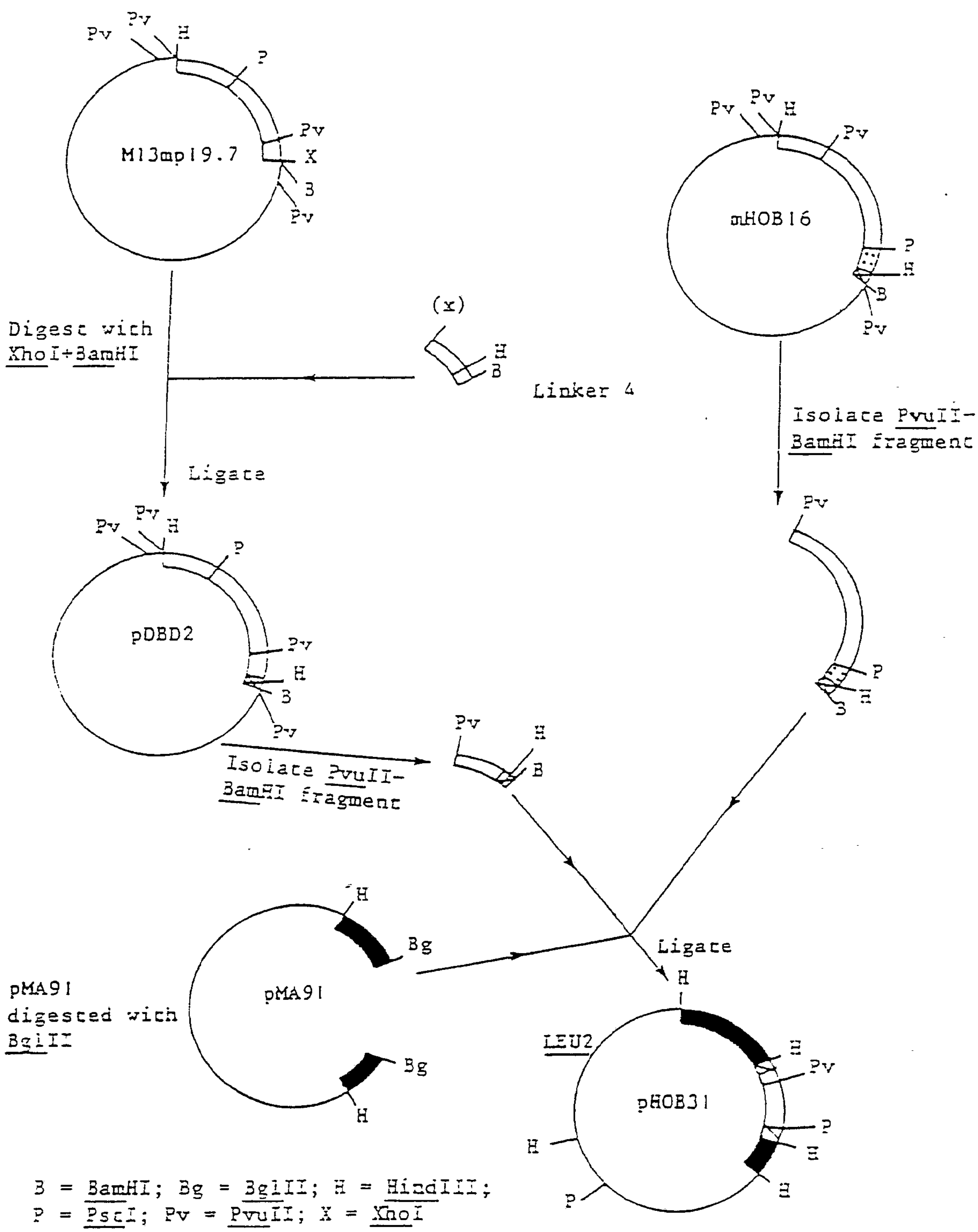
```
      1050      1060      1070      1080      1090      1100      1110      1120
GAGACTTGCCAAGACATATGAAACCACTCTAGAGAAGTGCTGTGCCGCTGCAGATCCTCATGAATGCTATGCCAAAGTGT
 R  L  A  K  T  Y  E  T  T  L  E  K  C  C  A  A  A  D  P  H  E  C  Y  A  K  V

      1130      1140      1150      1160      1170      1180      1190      1200
TCGATGAATTTAAACCTCTTGTGGAAGAGCCTCAGAATTTAATCAAACAAAACTGTGAGCTTTTTGAGCAGCTTGGAGAG
F  D  E  F  K  P  L  V  E  E  P  Q  N  L  I  K  Q  N  C  E  L  F  E  Q  L  G  E

      1210      1220      1230      1240      1250      1260      1270      1280
TACAAATTCCAGAATGCGCTATTAGTTCGTTACACCAAGAAAGTACCCCAAGTGTCAACTCCAACTCTTGTAGAGGTCTC
 Y  K  F  Q  N  A  L  L  V  R  Y  T  K  K  V  P  Q  V  S  T  P  T  L  V  E  V  S

      1290      1300      1310      1320      1330      1340      1350      1360
AAGAAACCTAGGAAAAGTGGGCAGCAAATGTTGTAAACATCCTGAAGCAAAAAGAATGCCCTGTGCAGAAGACTATCTAT
  R  N  L  G  K  V  G  S  K  C  C  K  H  P  E  A  K  R  M  P  C  A  E  D  Y  L

      1370      1380      1390      1400      1410      1420      1430      1440
CCGTGGTCCTGAACCAGTTATGTGTGTTGCATGAGAAAACGCCAGTAAGTGACAGAGTCACAAAATGCTGCACAGAGTCC
S  V  V  L  N  Q  L  C  V  L  H  E  K  T  P  V  S  D  R  V  T  K  C  C  T  E  S

      1450      1460      1470      1480      1490      1500      1510      1520
TTGGTGAACAGGCGACCATGCTTTTCAGCTCTGGAAGTCGATGAAACATACGTTCCCAAAGAGTTTAATGCTGAAACATT
 L  V  N  R  R  P  C  F  S  A  L  E  V  D  E  T  Y  V  P  K  E  F  N  A  E  T  F

      1530      1540      1550      1560      1570      1580      1590      1600
CACCTTCCATGCAGATATATGCACACTTTCTGAGAAGGAGAGACAAATCAAGAAACAAACTGCACTTGTTGAGCTTGTGA
  T  F  H  A  D  I  C  T  L  S  E  K  E  R  Q  I  K  K  Q  T  A  L  V  E  L  V

      1610      1620      1630      1640      1650      1660      1670      1680
AACACAAGCCCAAGGCAACAAAAGAGCAACTGAAAGCTGTTATGGATGATTTCGCAGCTTTTGTAGAGAAGTGCTGCAAG
K  H  K  P  K  A  T  K  E  Q  L  K  A  V  M  D  D  F  A  A  F  V  E  K  C  C  K

      1690      1700      1710      1720      1730      1740      1750      1760
GCTGACGATAAGGAGACCTGCTTTGCCGAGGAGGGTAAAAAACTTGTTGCTGCAAGTCAAGCTGCCTTAGGCTTATAACA
 A  D  D  K  E  T  C  F  A  E  E  G  K  K  L  V  A  A  S  Q  A  A  L  G  L

      1770      1780
TCTACATTTAAAAGCATCTCAG
```

FIGURE 3 Construction of mHOB16



B = BamHI; E = EcoRI; H = HindIII;
Hc = HincII; P = PstI; X = XhoI

FIGURE 4 Construction of pHOB31



B = BamHI; Bg = BglII; H = HindIII;
P = PstI; Pv = PvuII; X = XhoI

Gln Ala Gln Gln Met Val Gln Pro Gln 10 Ser Pro Val Ala Val Ser Gln Ser Lys Pro 20 Gly
Cys Tyr Asp Asn Gly Lys His Tyr Gln 30 Ile Asn Gln Gln Trp Glu Arg Thr Tyr Leu 40 Gly
Asn Val Leu Val Cys Thr Cys Tyr Gly 50 Gly Ser Arg Gly Phe Asn Cys Glu Ser Lys 60 Pro
Glu Ala Glu Glu Thr Cys Phe Asp Lys 70 Tyr Thr Gly Asn Thr Tyr Arg Val Gly Asp 80 Thr
Tyr Glu Arg Pro Lys Asp Ser Met Ile 90 Trp Asp Cys Thr Cys Ile Gly Ala Gly Arg 100 Gly
Arg Ile Ser Cys Thr Ile Ala Asn Arg 110 Cys His Glu Gly Gly Gln Ser Tyr Lys Ile 120 Gly
Asp Thr Trp Arg Arg Pro His Glu Thr 130 Gly Gly Tyr Met Leu Glu Cys Val Cys Leu 140 Gly
Asn Gly Lys Gly Glu Trp Thr Cys Lys 150 Pro Ile Ala Glu Lys Cys Phe Asp His Ala 160 Ala
Gly Thr Ser Tyr Val Val Gly Glu Thr 170 Trp Glu Lys Pro Tyr Gln Gly Trp Met Met 180 Val
Asp Cys Thr Cys Leu Gly Glu Gly Ser 190 Gly Arg Ile Thr Cys Thr Ser Arg Asn Arg 200 Cys
Asn Asp Gln Asp Thr Arg Thr Ser Tyr 210 Arg Ile Gly Asp Thr Trp Ser Lys Lys Asp 220 Asn
Arg Gly Asn Leu Leu Gln Cys Ile Cys 230 Thr Gly Asn Gly Arg Gly Glu Trp Lys Cys 240 Glu
Arg His Thr Ser Val Gln Thr Thr Ser 250 Ser Gly Ser Gly Pro Phe Thr Asp Val Arg 260 Ala
Ala Val Tyr Gln Pro Gln Pro His Pro 270 Gln Pro Pro Pro Tyr Gly His Cys Val Thr 280 Asp
Ser Ely Val Val Tyr Ser Val Gly Met 290 Gln Trp Leu Lys Thr Gln Gly Asn Lys Gln 300 Met
Leu Cys Thr Cys Leu Gly Asn Gly Val 310 Ser Cys Gln Glu Thr Ala Val Thr Gln Thr 320 Tyr
Gly Gly Asn Ser Asn Gly Glu Pro Cys 330 Val Leu Pro Phe Thr Tyr Asn Gly Arg Thr 340 Phe
Tyr Ser Cys Thr Thr Glu Gly Arg Gln 350 Asp Gly His Leu Trp Cys Ser Thr Thr Ser 360 Asn
Tyr Glu Gln Asp Gln Lys Tyr Ser Phe 370 Cys Thr Asp His Thr Val Leu Val Gln Thr 380 Gln
Gly Gly Asn Ser Asn Ely Ala Leu Cys 390 His Phe Pro Phe Leu Tyr Asn Asn His Asn 400 Tyr
Thr Asp Cys Thr Ser Glu Gly Arg Arg 410 Asp Asn Met Lys Trp Cys Gly Thr Thr Gln 420 Asn

Fig. 5A

Tyr Asp Ala Asp Gln Lys Phe Gly Phe 430 Cys Pro Met Ala Ala His Glu Glu Ile Cys 440 Thr

Thr Asn Glu Gly Val Met Tyr Arg Ile 450 Gly Asp Gln Trp Asp Lys Gln His Asp Met 460 Gly

His Met Met Arg Cys Thr Cys Val Gly 470 Asn Gly Arg Gly Glu Trp Thr Cys Tyr Ala 480 Tyr

Ser Gln Leu Arg Asp Gln Cys Ile Val 490 Asp Asp Ile Thr Tyr Asn Val Asn Asp Thr 500 Phe

His Lys Arg His Glu Glu Gly His Met 510 Leu Asn Cys Thr Cys Phe Gly Gln Gly Arg 520 Gly

Arg Trp Lys Cys Asp Pro Val Asp Gln 530 Cys Gln Asp Ser Glu Thr Gly Thr Phe Tyr 540 Gln

Ile Gly Asp Ser Trp Glu Lys Tyr Val 550 His Gly Val Arg Tyr Gln Cys Tyr Cys Tyr 560 Gly

Arg Gly Ile Gly Glu Trp His Cys Gln 570 Pro Leu Gln Thr Tyr Fro Ser Ser Ser Gly 580 Pro

Val Elu Val Phe Ile Thr Glu Thr Pro 590 Ser Gln Pro Asn Ser His Pro Ile Gln Trp 600 Asn

Ala Pro Gln Pro Ser His Ile Ser Lys 610 Iyr Ile Leu Arg Trp Arg Pro Lys Asn Ser 620 Val

Gly Arg Trp Lys Glu Ale Thr Ile Pro 630 Gly His Leu Asn Ser Tyr Thr Ile Lys Gly 640 Leu

Lys Pro Gly Val Val Tyr Glu Gly Glr 650 Leu Ile Ser Ile Gln Gln Tyr Gly His Gln 660 Glu

Val Thr Arg Phe Asp Phe Thr Thr Thr 670 Ser Thr Ser Thr Pro Val Thr Ser Asn Thr 680 Val

Thr Gly Glu Thr Thr Pro Phe Ser Pro 690 Leu Val Ala Thr Ser Glu Ser Val Thr Glu 700 Ile

Thr Ala Ser Ser Phe Val Val Ser Trp 710 Val Ser Ala Ser Asp Thr Val Ser Gly Phe 720 Arg

Val Glu Tyr Glu Leu Ser Glu Glu Gly 730 Asp Glu Pro Gln Tyr Leu Asp Leu Pro Ser 740 Thr

Ala Thr Ser Val Asn Ile Pro Asp Leu 750 Leu Pro Ely Arg Lys Tyr Ile Val Asn Val 760 Tyr

Gln Ile Ser Glu Asp Gly Glu Gln Ser 770 Leu Ile Leu Ser Thr Ser Gln Thr Thr Ala 780 Pro

Asp Ala Pro Pro Asp Pro Thr Val Asp 790 Gln Val Asp Asp Thr Ser Ile Val Val Arg 800 Trp

Ser Arg Pro Glr Ala Pro Ile Thr Gly 810 Tyr Arg Ile Val Tyr Ser Pro Ser Val Glu 820 Gly

Ser Ser Thr Glu Leu Asn Leu Pro Glu 830 Thr Ala Asn Ser Val Thr Leu Ser Asp Leu 840 Gln

FNDEL 1

Fig. 5B

850 860
Pro Gly Val Gln Tyr Asn Ile Thr Ile Tyr Ala Val Glu Glu Asn Gln Glu Ser Thr Pro

870 880
Val Val Ile Gln Gln Glu Thr Thr Gly Thr Pro Arg Ser Asp Thr Val Pro Ser Pro Arg

890 900
Aso Leu Gln Phe Val Glu Val Thr Asp Val Lys Val Thr Ile Met Trp Thr Pro Pro Glu

910 920
Ser Ala Val Thr Gly Tyr Arg Val Asp Val Ile Pro Val Asn Leu Pro Gly Glu His Gly

930 940
Gln Arg Leu Pro Ile Ser Arg Asn Thr Phe Ala Glu Val Thr Gly Leu Ser Pro Gly Val

950 960
Thr Tyr Tyr Phe Lys Val Phe Ala Val Ser His Gly Arg Glu Ser Lys Pro Leu Thr Ala

970 980
Gln Gln Thr Thr Lys Leu Asp Ala Pro Thr Asn Leu Gln Phe Val Asn Glu Thr Asp Ser

990 1000
Thr Val Leu Val Arg Trp Thr Pro Pro Arg Ala Gln Ile Thr Gly Tyr Arg Leu Thr Val

1010 1020
Gly Leu Thr Arg Arg Gly Gln Pro Arg Gln Tyr Asn Val Gly Pro Ser Val Ser Lys Tyr

1030 1040
Pro Leu Arg Asn Leu Gln Pro Ala Ser Glu Tyr Thr Val Ser Leu Val Ala Ile Lys Gly

1050 1060
Asn Gln Glu Ser Pro Lys Ala Thr Gly Val Phe Thr Thr Leu Gln Pro Gly Ser Ser Ile

1070 1080
Pro Pro Tyr Asn Thr Glu Val Thr Glu Thr Thr Ile Val Ile Thr Trp Thr Pro Ala Pro

1090 1100
Arg Ile Gly Phe Lys Leu Gly Val Arg Pro Ser Gln Gly Gly Glu Ala Pro Arg Glu Val

1110 1120
Thr Ser Asp Ser Gly Ser Ile Val Val Ser Gly Leu Thr Pro Gly Val Glu Tyr Val Tyr

1130 1140
Thr Ile Gln Val Leu Arg Asp Gly Gln Glu Arg Asp Ala Pro Ile Val Asn Lys Val Val

1150 1160
Thr Pro Leu Ser Pro Pro Thr Asn Leu His Leu Glu Ala Asn Pro Asp Thr Gly Val Leu

1170 1180
Thr Val Ser Trp Glu Arg Ser Thr Thr Pro Asp Ile Thr Gly Tyr Arg Ile Thr Thr Thr

1190 1200
Pro Thr Asn Gly Gln Gln Gly Asn Ser Leu Glu Glu Val Val His Ala Asp Gln Ser Ser

1210 1220
Cys Thr Phe Asp Asn Leu Ser Pro Gly Leu Glu Tyr Asn Val Ser Val Tyr Thr Val Lys

1230 1240
Asp Asp Lys Glu Ser Val Pro Ile Ser Asp Thr Ile Ile Pro Ala Val Pro Pro Pro Thr

1250 1260
Asp Leu Arg Phe Thr Asn Ile Gly Pro Asp Thr Met Arg Val Thr Trp Ala Pro Pro Pro

Fig. 5C

Fig. 5D

Ser Ile Asp Leu Thr Asn Phe Leu Val 1270 Arg Tyr Ser Pro Val Lys Asn Glu Glu Asp 1280 Val
Ala Glu Leu Ser Ile Ser Pro Ser Asp 1290 Asn Ala Val Val Leu Thr Asn Leu Leu Pro 1300 Gly
Thr Glu Tyr Val Val Ser Val Ser Ser 1310 Val Tyr Glu Gln His Glu Ser Thr Pro Leu 1320 Arg
Gly Arg Gln Lys Thr Gly Leu Asp Ser 1330 Pro Thr Gly Ile Asp Phe Ser Asp Ile Thr 1340 Ala
Asn Ser Phe Thr Val His Trp Ile Ala 1350 Pro Arg Ala Thr Ile Thr Gly Tyr Arg Ile 1360 Arg
His His Pro Glu His Phe Ser Gly Arg 1370 Pro Arg Glu Asp Arg Val Pro His Ser Arg 1380 Asn
Ser Ile Thr Leu Thr Asn Lau Thr Pro 1390 Gly Thr Glu Tyr Val Val Ser Ile Val Ala 1400 Leu
Asn Gly Arg Glu Glu Ser Pro Leu Leu 1410 Ile Gly Gln Gln Ser Thr Val Ser Asp Val 1420 Pro
Arg Asp Leu Glu Val Val Ala Ala Thr 1430 Pro Thr Ser Leu Leu Ile Ser Trp Asp Ala 1440 Pro
Ala Val Thr Val Arg Tyr Tyr Arg Ile 1450 Thr Tyr Gly Glu Thr Gly Gly Asn Ser Pro 1460 Val
Gln Glu Phe Thr Val Pro Gly Ser Lys 1470 Ser Thr Ala Thr Ile Ser Gly Leu Lys Pro 1480 Gly
Val Asp Tyr Thr Ile Thr Val Tyr Ala 1490 Val Thr Gly Arg Gly Asp Ser Pro Ala Ser 1500 Ser
Lys Pro Ile Ser Ile Asn Tyr Arg Thr 1510 Glu Ile Asp Lys Pro Ser Gln Met Gln Val 1520 Thr
Asp Val Gln Asp Asn Ser Ile Ser Val 1530 Lys Trp Leu Pro Ser Ser Ser Pro Val Thr 1540 Gly
Tyr Arg Val Thr Thr Thr Pro Lys Asn 1550 Gly Pro Gly Pro Thr Lys Thr Lys Thr Ala 1560 Gly
Pro Asp Gln Thr Glu Met Thr Ile Glu 1570 Gly Leu Gln Pro Thr Val Glu Tyr Val Val 1580 Ser
Val Tyr Ala Gln Asn Pro Ser Gly Glu 1590 Ser Gln Pro Leu Val Gln Thr Ala Val Thr 1600 Thr
Ile Pro Ala Pro Thr Asp Leu Lys Phe 1610 Thr Gln Val Thr Pro Thr Ser Leu Ser Ala 1620 Gln
Trp Thr Pro Pro Asn Val Gln Leu Thr 1630 Gly Tyr Arg Val Arg Val Thr Pro Lys Glu 1640 Lys
Thr Gly Pro Met Lys Glu Ile Asn Leu 1650 Ala Pro Asp Ser Ser Ser Val Val Val Ser 1660 Gly
Leu Met Val Ala Thr Lys Tyr Glu Val 1670 Ser Val Tyr Ala Leu Lys Asp Thr Leu Thr 1680 Ser
FNDEL 1

```
                                          1690                                                        1700
Arg Pro Ala Gln Gly Val Val Thr Thr Leu Glu Asn Val Ser Pro Pro Arg Arg Ala Arg
                                          1710                                                        1720
Val Thr Asp Ala Thr Glu Thr Thr Ile Thr Ile Ser Trp Arg Thr Lys Thr Glu Thr Ile
                                          1730                                                        1740
Thr Gly Phe Gln Val Asp Ala Val Pro Ala Asn Gly Gln Thr Pro Ile Gln Arg Thr Ile
                                          1750                                                        1760
Lys Pro Asp Val Arg Ser Tyr Thr Ile Thr Gly Leu Gln Pro Gly Thr Asp Tyr Lys Ile
                                          1770                                                        1780
Tyr Leu Tyr Thr Lau Asn Asp Asn Ala Arg Ser Ser Pro Val Val Ile Asp Ala Ser Thr
                                          1790                                                        1800
Ala Ile Asp Ala Pro Ser Asn Lau Arg Phe Lau Ala Thr Thr Pro Asn Ser Leu Leu Val
                                          1810                                                        1820
Ser Trp Gln Pro Pro Arg Ala Arg Ile Thr Gly Tyr Ile Ile Lys Tyr Glu Lys Pro Gly
                                          1830                                                        1840
Ser Pro Pro Arg Glu Val Val Pro Arg Pro Arg Pro Gly Val Thr Glu Ala Thr Ile Thr
                                          1850                                                        1860
Gly Leu Glu Pro Gly Thr Glu Tyr Thr Ile Tyr Val Ile Ala Leu Lys Asn Asn Gln Lys
                                          1870                                                        1880
Ser Glu Pro Leu Ile Gly Arg Lys Lys Thr Aso Glu Leu Pro Gln Leu Val Thr Leu Pro
                                          1890                                                        1900
His Pro Asn Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr Val Gln Lys Thr Pro
                                          1910                                                        1920
Phe Val Thr His Pro Gly Tyr Asp Thr Gly Asn Gly Ile Gln Leu Pro Gly Thr Ser Gly
                                          1930                                                        1940
Gln Gln Pro Ser Val Gly Gln Gln Met Ile Phe Glu Glu His Gly Phe Arg Arg Thr Thr
                                          1950                                                        1960
Pro Pro Thr Thr Ala Thr Pro Ile Arg His Arg Pro Arg Pro Tyr Pro Pro Asn Val Ala
                                          1970                                                        1980
Leu Ser Gln Thr Thr Ile Ser Trp Ala Pro Phe Gln Aso Thr Ser Glu Tyr Ile Ile Ser
                                          1990                                                        2000
Cys His Pro Val Gly Thr Asp Glu Glu Pro Leu Gln Phe Arg Val Pro Gly Thr Ser Thr
                                          2010                                                        2020
Ser Ala Thr Leu Thr Gly Leu Thr Arg Gly Ala Thr Tyr Asn Ile Ile Val Glu Ala Leu
                                          2030                                                        2040
Lys Asp Gln Gln Arg His Lys Val Arg Elu Glu Val Val Thr Val Gly Asn Ser Val Asn
                                          2050                                                        2060
Glu Gly Leu Asn Gln Pro Thr Asp Asp Ser Cys Phe Asp Pro Tyr Thr Val Ser His Tyr
                                          2070                                                        2080
Ala Val Gly Asp Glu Trp Glu Arg Met Ser Glu Ser Gly Phe Lys Leu Leu Cys Gln Cys
                                          2090                                                        2100
Leu Ser Phe Gly Ser Gly His Phe Arg Cys Asp Ser Ser Arg Trp Cys His Asp Asn Gly
```

Fig. 5E

```
                                    2110                                         2120
Val Asn Tyr Lys Ile Gly Glu Lys Trp Asp Arg Gln Gly Glu Asn Gly Gln Met Met Ser
                                    2130                                         2140
Cys Thr Cys Leu Gly Asn Gly Lys Gly Glu Phe Lys Cys Asp Pro His Glu Ala Thr Cys
                                    2150                                         2160
Tyr Asp Asp Gly Lys Thr Tyr His Val Gly Glu Gln Trp Gln Lys Glu Tyr Leu Gly Ala
                                    2170                                         2180
Ile Cys Ser Cys Thr Cys Phe Gly Gly Gln Arg Gly Trp Arg Cys Asp Asn Cys Arg Arg
                                    2190                                         2200
Pro Gly Gly Glu Pro Ser Pro Glu Gly Thr Thr Gly Gln Ser Tyr Asn Gln Tyr Ser Gln
                                    2210                                         2220
Arg Tyr His Gln Arg Thr Asn Thr Asn Val Asn Cys Pro Ile Glu Cys Phe Met Pro Leu
                                    2230
Asp Val Gln Ala Asp Arg Glu Asp Ser Arg Glu
```

Fig. 5F

```
                      1                                       2
GAAGAGCCTCAGAATTTAATCACTGAGACTCCGAGTCAGCCCAACTCCCACCCCATCCAGTGG
CTTCTCGGAGTCTTAAATTAGTGACTCTGAGGCTCAGTCGGGTTGAGGGTGGGGTAGGTCACC
e  e  p  q  n  l  i  t  e |t  p  s  q  p  n  s  h  p  i  q  w
                          8

                                                       3
AATGCACCACAGCCATCTCACATTTCCAAGTACATTCTCAGGTGGAGACCTAAAAATTCTGTA
TTACGTGGTGTCGGTAGAGTGTAAAGGTTCATGTAAGAGTCCACCTCTGGATTTTTAAGACAT
n  a  p  q  p| s  h  i  s  k  y  i  l  r  w  r  p  k  n  s  v
            7

                                    4
GGCCGTTGGAAGGAAGCTACCATACCAGGCCACTTAAACTCCTACACCATCAAAGGCCTG
CCGGCAACCTTCCTTCGATGGTATGGTCCGGTGAATTTGAGGATGTGGTAGTTTCCGGACTTAA
g| r  w  k  e  a  t  i  p  g  h  l  n  s| y  t  i  k  g  l
 6                                       5
```

Figure 6 Linker 5 showing the eight constituent oligonucleotides

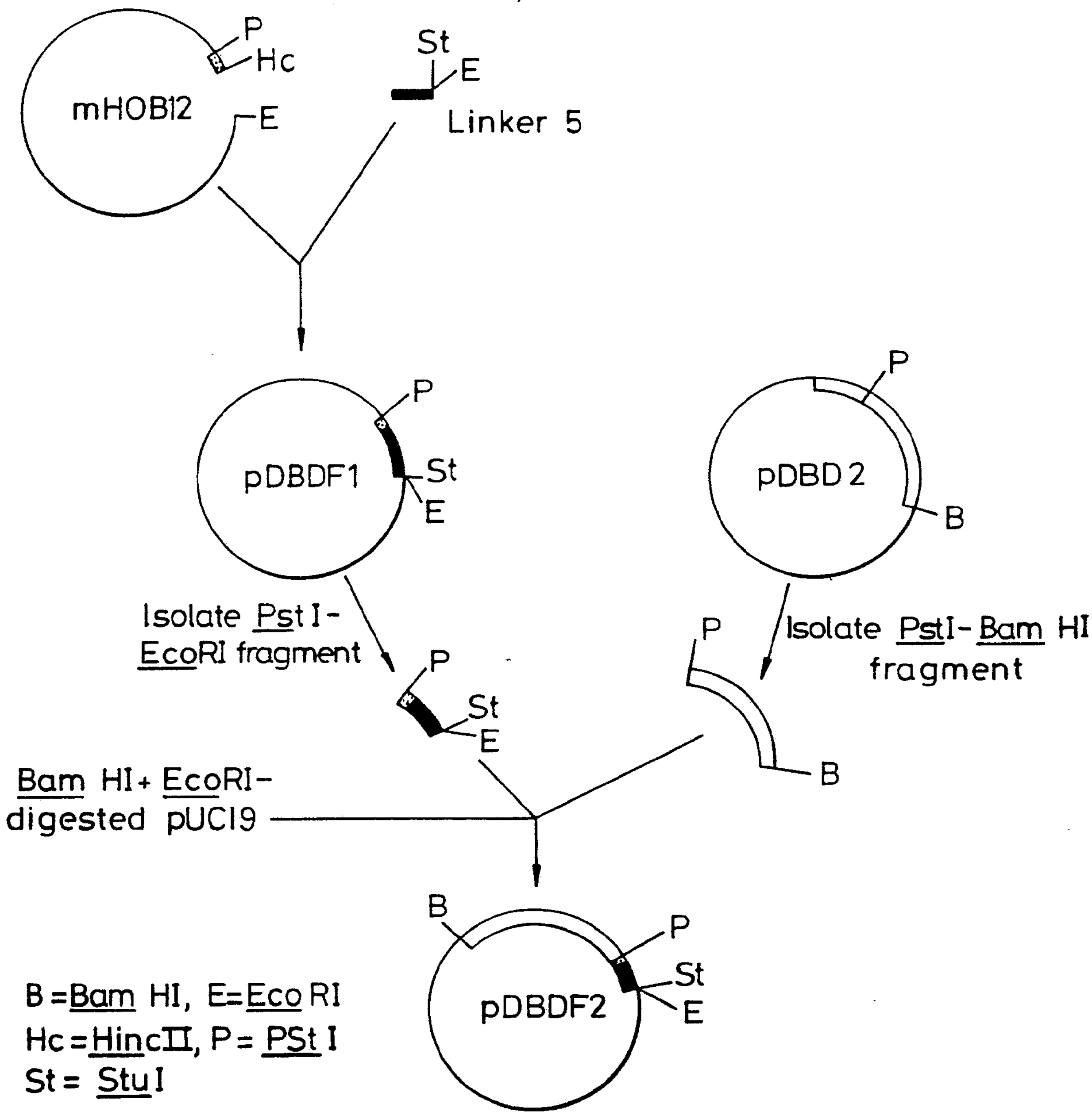


Fig. 7 Construction of pDBDF2

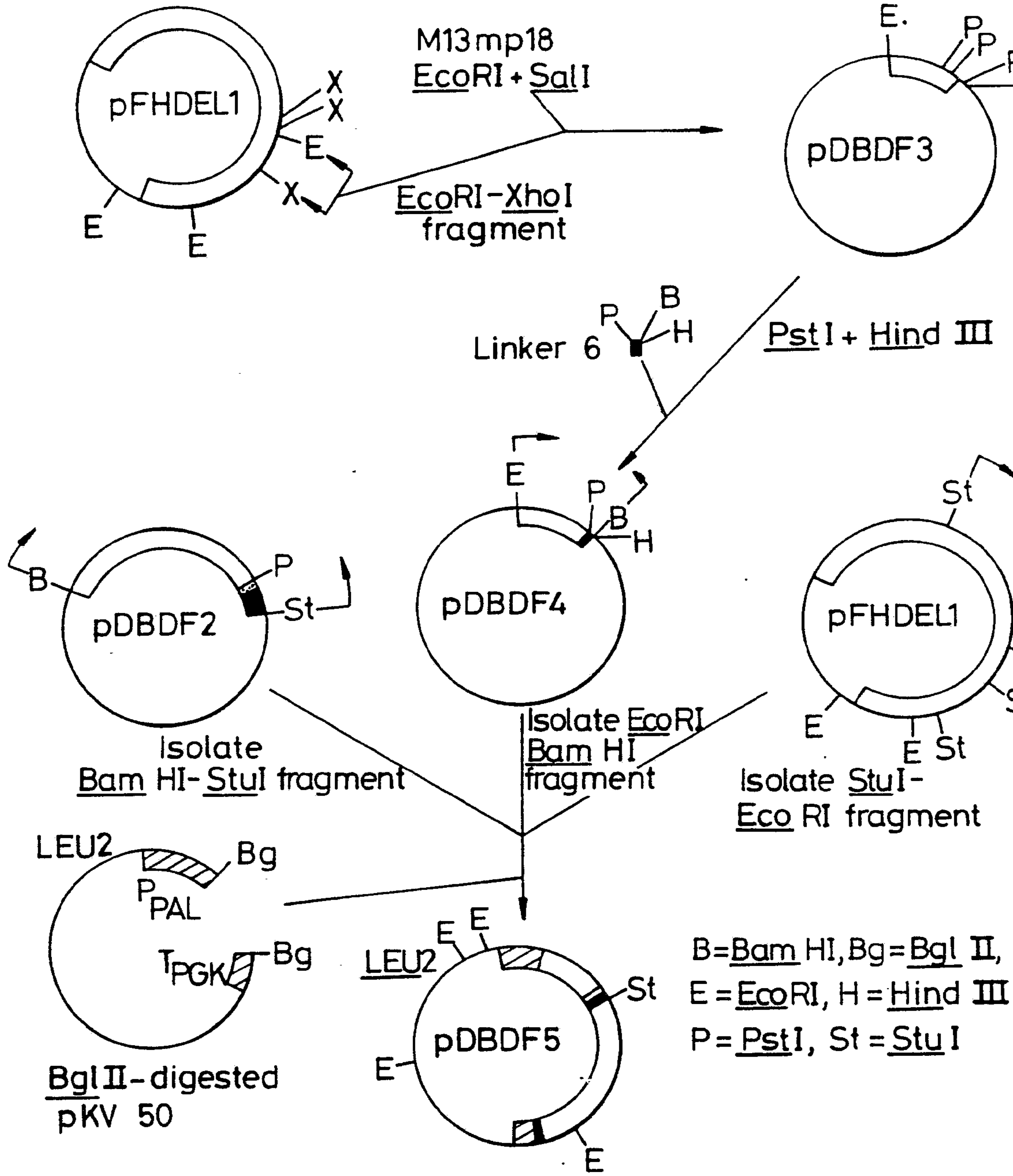


Fig. 8 Construction of pDBDF5

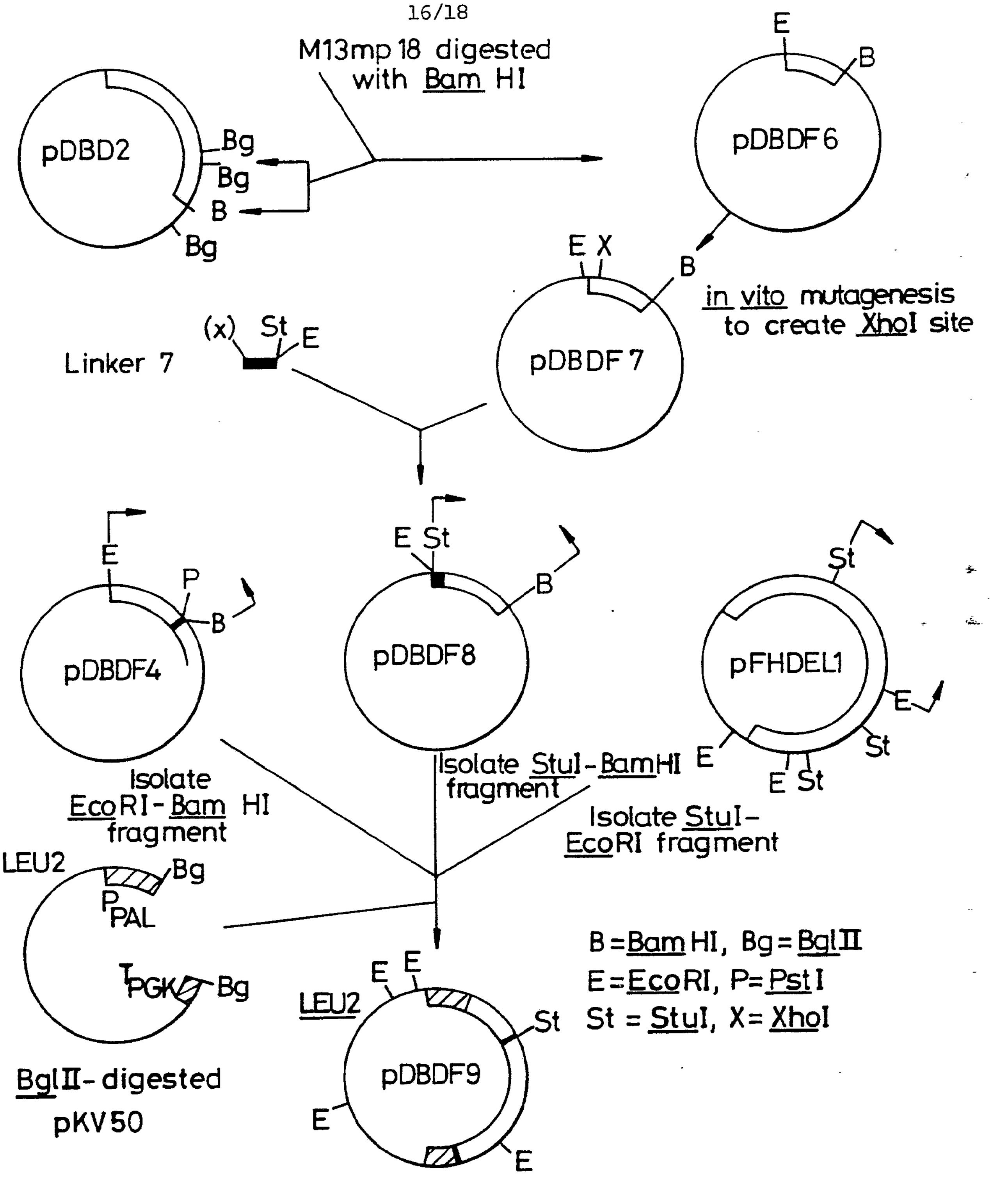


Fig. 9 Construction of pDBDF9

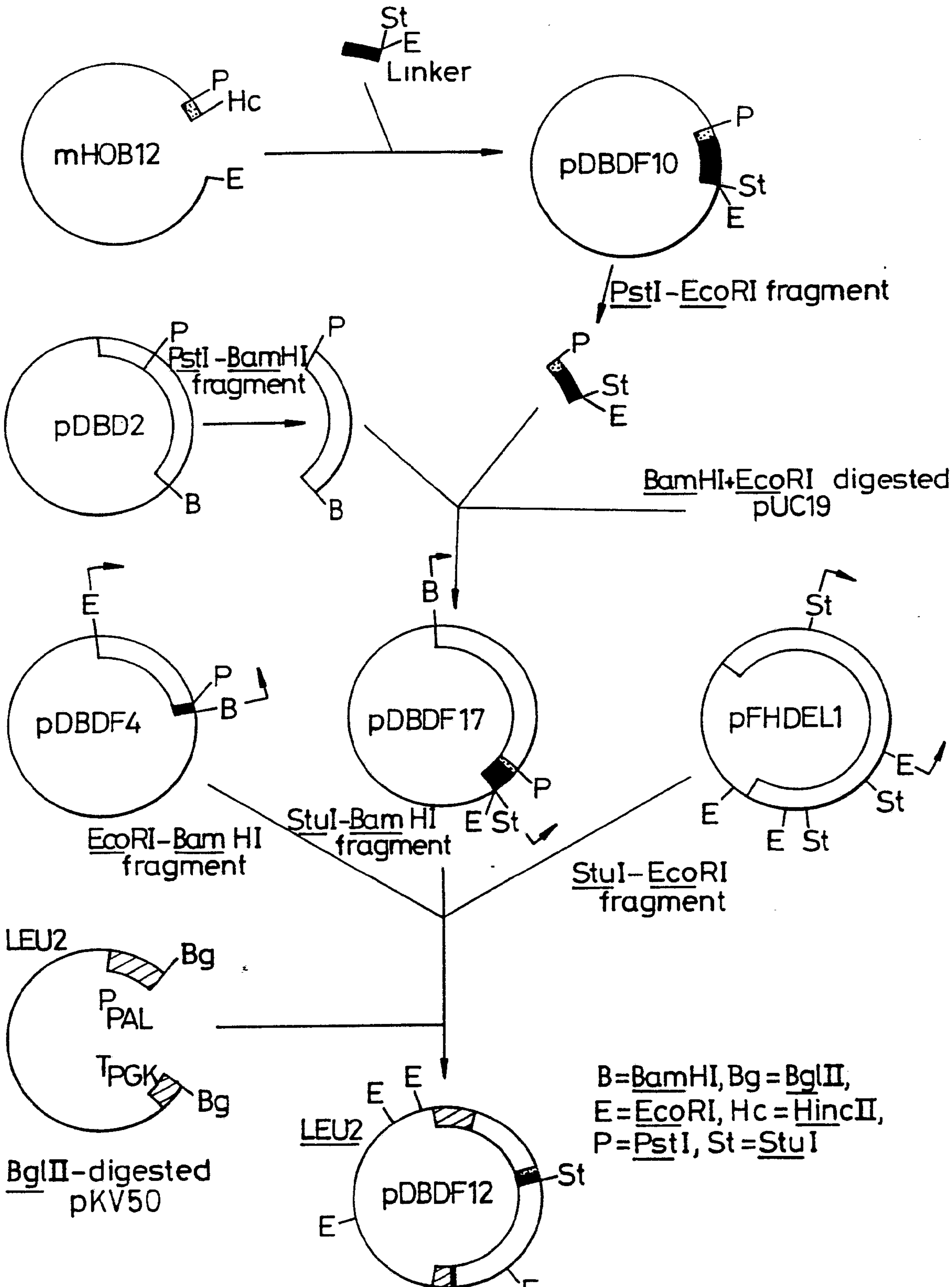
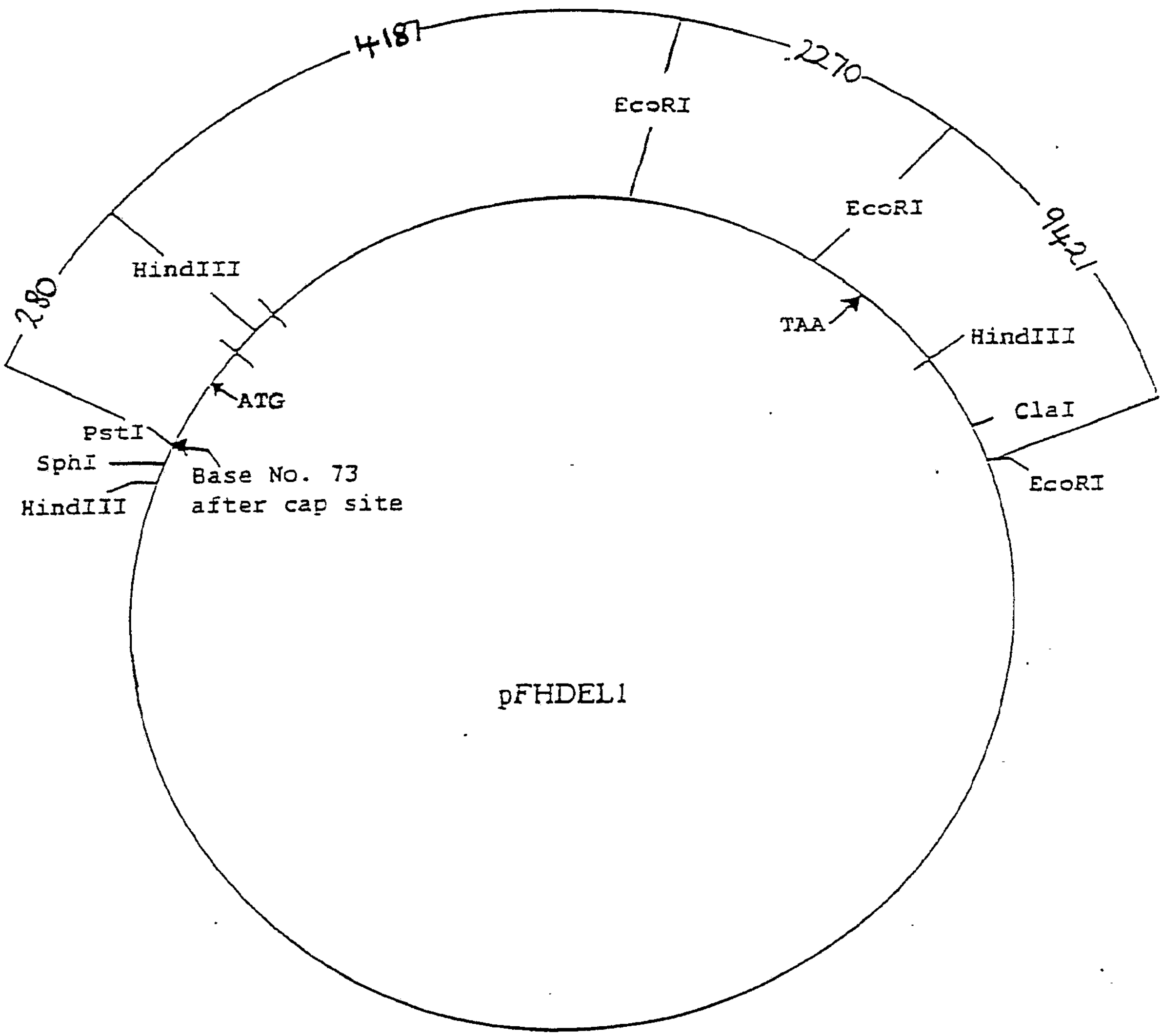


Fig. 10 Construction of pDBDF12

Figure 11

Name: pFHDEL1
Vector: pUC18 Amp^{fy} 2860bp
Insert: hFNcDNA ~ 7630bp



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00650

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 12 N 15/62, C 07 K 13/00, C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N, C 12 P, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A, 0308381 (SKANDIGEN et al.) 22 March 1989	
	--	
T	EP, A, 0322094 (DELTA BIOTECHNOLOGY LTD) 28 June 1989 (cited in the application)	
	--------	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th July 1990	09.08.90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. SOTELO

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9000650 SA 36670

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 31/07/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0308381	22-03-89	SE-B- 459586	17-07-89
		AU-A- 2420488	17-04-89
		SE-A- 8703539	15-03-89
		WO-A- 8902467	23-03-89
EP-A- 0322094	28-06-89	AU-A- 2404688	18-05-89

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82